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The Potential Use of the Eustigmatophyceae In the Production of Biofuels

Potenciál využití eustigmatofytních řas v produkci biopaliv

UNDERGRADUATE THESIS

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu.Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

The eustigmatophytes – yellow-green microalgae – are well known for their high lipid content which is constituted by high amounts of essential polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA). This, combined with their high growth rate and an exceptionally high adaptability, makes them very promising feedstock for the biofuels production. In this bachelor thesis the role of eustigmatophytes in the production of biofuels is depicted with consideration of the issue of various cultivation conditions effects on the lipid content and composition. The first review of fatty acid distribution in eustigmatophytes ever reported is provided herein. Thus this work might be tentatively contributing to the strain selection issue – one of the most discussed topics in the biofuels field. The fatty acid distribution is qualitatively and quantitatively changed under various chemical and physical stress conditions. Thus the impact of nitrogen and phosphorous availability alongside with light intensity or temperature on lipid changes are presented. In addition, the process of fatty acid and polyunsaturated fatty acid synthesis is briefly outlined. Recent genetic engineering trends and successes are also mentioned, such as the implementation of high-efficiency homologous recombination to eustigmatophytes.

Keywords: biofuels, eicosapentaenoic acid, Eustigmatophyceae, fatty acid profile, *Monodopsis*, *Nannochloropsis*, nutrient deprivation, polyunsaturated fatty acids, *Trachydiscus*, *Vischeria*

Abstrakt

Eustigmatophyta jsou žlutozelené řasy dobře známé vysokým obsahem lipidů. Tyto lipidy mají vyšší obsah esenciálních vícenenasycených mastných kyselin (PUFA), z toho především kyseliny eikosapentaenové (EPA). Další výhodou těchto řas je vysoká rychlost růstu. Navíc jsou schopné se velmi dobře přizpůsobt podmínkám prostředí, ve kterém žijí. To vše přispívá k tomu, že je lze využít jako zdroj biopaliv nebo pro jiné biotechnologické účely. Vpředložené bakalářské práci je rozebírán potenciál eustigmatophyt v produkci biopaliv. Je diskutován vliv prostředí jak na množství obsažených lipidů, tak i na jejich složení. Tato práce poskytuje ucelený přehled mastných kyselin obsažených v eustigmatofytech a může napomoci při výběru vhodného kmene pro další experimenty. Obsahy mastných kyselin se kvalitativně i kvantitativně liší v různých chemicky nebo fyzikálně vyvolaných stresových podmínkách. Některé tyto vlivy, jako např. dostupnost dusíku a fosforu spolu s vlivem teploty a světelné intenzity, jsou diskutovány podrobněji. Dále je stručně nastíněn proces biosyntézy mastných kyselin včetně vícenenasycených mastných kyselin. Na závěr jsou zmíněny současné trendy a úspěchy genového inženýrství v souvislosti s eustigmatophytními řasami, jako je úspěšné provedení homologní rekombinace.

Klíčová slova: biopaliva, eicksapentaenová kyselina, efekt nedostatku živin, Eustigmatophyceae, profil mastných kyselin, *Monodopsis*, *Nannochloropsis*, *Trachydiscus*, vícenenasycené mastné kyseliny, *Vischeria*

List of abbrevitaions

ACCase	acetyl-CoA carboxylase
ALA	α-linolenic acid
aLRT	approximate likelihood-ratio test for branches
ARA	arachidonic acid
ATP	adenosine triphosphate
BODIPY	boron-dipyrromethene
CCAP	Culture Collection of Algae and Protozoa
CoA	coenzyme A
DAG	diacylglycerols
DAGAT	diacylglcyerol acyltransferase
DGDG	digalactosyl diacylglycerols
DGTS	diacylglycerol trimethyl homoserine
DHA	docosahexaenoic acid
d.w.	dry weight
EPA	eicosapentaenoic acid
ETC	electron transport chain
FA	fatty acid
FAS	fatty acid synthase
GC-MS	gas chromatography-mass spectrometry
GLA	γ-linolenic acid
HPLC	high-performance liquid chromatography
LC-PUFA	long chain polyunsaturated fatty acids
MGDG	monogalactosyl diacylglycerols
NADPH	nicotinamide adenine dinucleotide phosphate
NIVA	Norwegian Institute for Water Research (the culture collection of algae)
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PDAT	phospholipid:DAG acyltransferase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKS	polyketide synthase
PUFA	polyunsaturated fatty acids
SAG	culture collection of microalgae in Göttingen (Germany)
SFA	saturated fatty acids
SQDG	sulphoquinovosyl diacylglycerol
TAG	triacylglycerols
TEM	transmission electron microscopy
TFA	total fatty acids
UTEX	Culture Collection of Algae at the University of Texas in Austin

Contents

1 Introduction

There are over 30 000 different species of microalgae which contain a variety of unique chemicals, some of which are of commercial value. Almost all the articles reporting on lipid content in algae and their other biochemical features begin with, or at least mention, some of the impressive benefits of the range of chemicals, which can be isolated in commercially significant quantities and applied in the production of biofuels, cosmetics, pharmaceuticals, nutrition and food additives or aquaculture, while helping the environment by the pollution prevention in wastewater treatment, effective land utilization or by biological sequestration of carbon dioxide, all of that without triggering food versus fuel feud (Cohen, 1999; Lam and Lee, 2012; Mata et al., 2010 and references therein). The mantra of renewable resources, sustainability, ecology, cleanness, saving the world against global warming and fossil fuels exhaustion is always repeated in there but without the deep research which is necessary for all the problems connected with the sustainability and economical viability of microalgae cultivations on a large scale to be finally solved.

1.1 Biotechnological applications

Since there is a rapidly increasing interest in sustainable technologies to produce different commodity for human consumption we can also follow the rapid development in algal biotechnologies even in such a small class of the Eustigmatophyceae. The eustigmatophytes are well known for their high lipid content (Fig. 2), which is constituted by high amounts of essential polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA, Table 1, 2). This is combined with a high growth rate – a hundred times higher than terrestrial plants with the ability to double their biomass in less than one day – and finally they have an exceptionally high adaptability towards surrounding environment. All together those characteristics make them a very promising target for various biotechnological applications ranging from biofuels production to extraction of long-chain polyunsaturated fatty acids (LC-PUFA) for nutraceuticals and pharmaceuticals (Lam and Lee, 2012). Nevertheless they (e.g. *Eustigmatos* cf. *polyphem*, *Nannochloropsis*, *Trachydiscus minutus*) are also a promising source of β-carotenes, i.e. provitamin A with significant physiological effects, such as the stimulantion of immune response, being an antioxidant and a compound necessary for good vision. Apart from that, β-carotene can also be used as a colorant, feed additive, anti-cancer agent and heart disease preventive (Li et al., 2012; Pilát et al., 2011). *Nannochloropsis* is also well known as a source of pigments, such as chlorophyll *a*, zeaxanthin, canthaxanthin, and astaxanthin (Sukenik 1999). In addition, eustigmatophytes are rich in vitamins B, C, D and K (Liu and Lin, 2005).

PUFA are almost exclusively synthesized by algae and plants. Animals can convert them from one form to another through elongation and desaturation, but very few can synthesize PUFA *de novo*. PUFA play an important role in regulating cell membrane properties and serve as precursors for important animal hormones and are essential for animals (Brett and Müller-Navara, 1997). Long-chain PUFA (LC-PUFA) are even used in the prevention and treatment of chronic diseases, such as coronary heart disease, hypertension, type II diabetes, ocular diseases, arthritis and cystic

fibrosis (Simopoulos, 1999). Particularly, docosahexaenoic acid (DHA) is essential for the normal functional development of the retina and brain in premature infants (Innis, 1992; Simopoulos, 1991).

The principal dietary source of DHA and EPA are fish, accumulating PUFA from their food, whereas the primary production is carried out mostly by microalgae. However, fish also accumulate pollutants, the extracted oil has unpleasant odour and the proportion of specific fatty acids in the lipids is difficult to control and the acids to extract (preparative HPLC is needed). In addition fish are a declining resource and there are serious environmental consequences related to the continued exploitation of fish-stocks in order to meet the demands of an expanding market. Because of these drawbacks, new sources of LC-PUFA are needed (Cohen, 1999; Tonon et al., 2002).

The cultivation of microalgae for EPA production has been developed and mainly applied in the aquaculture industry, where EPA is the preferred nutritional feed for zooplankton in artificial food chain for many fish hatchlings. In summary, PUFA contribute to the acceleration and increase of growth, reduction of mortality, improvement of fecundity, egg hatchability and the overall quality of the broodstock (Brett and Müller-Navara, 1997; Cavalli et al., 1999; Doroudi et al., 1999; Mata et al., 2010). Concomitantly, attempts have been made to use microalgal source of essential fatty acids as human diet supplements and animal feed (Sukenik, 1999). Poultry, cattle, pigs and even farmer fish are being fed by the algae or the lipid extracts from them, to achieve PUFA enrichment in their meat, milk or eggs. Such attempts have met with limited success. Furthermore, the cultivation of algae for LC-PUFA production is technically demanding and costly (Grima et al., 1995; Simopoulos, 2001; Tonon et al., 2002).

1.1.1 The biofuels production

There have been multiple different processes and procedures in the production of biofuels, which has gone through the development of various technical and conceptual approaches over the course of the time from the first to the third generation of biodiesel production from microalgae. For the production of first generation biodiesel, edible vegetable oils such as soybean, rapeseed, sunflower and palm oil have been used as the main feedstock and now it covers 99 % of all biodiesel production. However, the use of edible oils as energy source has raised a lot of objections and controversy because of the food and fuel feud, and its participation on the destruction of the world's forests, which are cut down in order to make space for planting of oil palms. Thus, second generation biodiesel derived from lignocellulosic agriculture and non-edible oils, such as *Jatropha curcas*, appeared at the same time as an attractive alternative feedstock for the biodiesel industry, as well as an inconvenient land occupant. Due to this weakness, the search for a more sustainable biodiesel feedstock continues and now focuses on microalgae as a third generation biodiesel source (as well as on yeast and fungi). According to a recent study (reported in the literature), a realistic value of microalgae biomass production lies between 15 and 25 tonne/ha/year. With an assumption of 30% lipid content in microalgae cells this is equivalent to a lipid production of 4,5–7,5 tonne/ha/year without optimizing the growth condition (e.g. in the species of *Nannochloropsis salina* the increase of total FA up to 70 % of the dry mass can be provoked). This amount is higher compared to the

production of oil from soybean (0,4 tonne/ha/year), rapeseed (0,68 tonne/ha/year), oil palm (3,62 tonne/ha/year) and jatropha (4,14 tonne/ha/year). Thus, culturing microalgae for biodiesel production requires the least land area (Hoffmann et al., 2010; Lam and Lee, 2012; Nigam and Singh, 2011; Tsukahara and Sawayama, 2005).

The history of algae usage in biotechnologies is thousands of years long and an idea of exploiting microalgae for biofuels production is not new either. In fact, it dates back to 1950s, when raceway ponds were first developed in Japan and Germany and after that closed systems of photobioreactors providing fully controlled algae culturing were invented. Then the algal biofuel technologies were spread into the USA, Australia, Czechoslovakia and other countries. The first attempts in culturing algae on a large scale in Czechoslovakia were made in late-1960s by Šetlík in Třeboň (Becker, 1993; Rodolfi et al., 2009; Šetlík et al., 1970).

Biodiesel is a mixture of fatty acid alkyl esters obtained by transesterification of plant and algal oils or animal fats (Mata et al., 2010). However, not all algal oils are compatible with the engines used at present. Biodiesels need to comply with existing standards, e.g. Standard EN 14214 in Europe, which is limiting the extent of total unsaturated FAs (iodine value), ALA methylester, the content of FAs with four and more double bonds and mono-, di- and triacylglycerol percentage (Griffiths et al., 2011; Rodolfi et al., 2009). PUFA are known to be responsible for poor volatility, low oxidation stability, and tendency for gum formation (Halim et al., 2011). One of possible solutions in case of algae containing high proportions of PUFA (e.g. the eustigmatophytes) is to separate them so that they could be used as a valuable nutraceutical (Olofsson et al., 2012). The ultimate aims for high quality biofuels production is a balanced fatty acid mixture of 16:1, 18:1, and 14:0 in the ratio of 5:4:1 (Schenk et al., 2008). This ideal mix has been identified as providing a good cetane number along with low oxidation potential and cold flow (Sivakumar et al., 2012).

Nevertheless, the production of biofuels from microalgae is also quite controversial. Algal biofuels production is not optimized on a really large scale, therefore it has not proved its sustainability and economic viability yet. The cost of biodiesel production should decrease ten times to be affordable and sustainable. To achieve that, it is suggested to use cheap source of CO_2 (flue gas) and nutrient-rich wastewater as an inexpensive fertilizer. Another important requirement for the production of biofuels is the easy adaptation in the existing biofuels industry, such as algal biodiesel compatibility with engines used at present (Lam and Lee, 2012; Mata et al., 2010; Schenk et al., 2008).

Consequently, there is also the approach of genetic engineering trying to control algal lipid and fatty acid production. Because none of the algae currently in use has a history of domestication, and bioengineering of algae is still in its infancy, there is a need to develop algal strains adapted to cultivation for industrial large-scale production of desired compounds (Radakovits et al., 2012).

1.2 The aims of the work

This bachelor thesis aims to determine the role of the Eustigmatophyceae in the production of biofuels in order to provide a wide perspective on lipids production and the variables determining the changes in fatty acid profiles. To achieve this objective, the following subgoals were identified:

- To introduce the class Eustigmatophyceae, briefly mentioning its morphological, ecological and molecular characteristics.
- To define the lipid classes and particular fatty acid profiles with the focus on polyunsaturated fatty acids in the Eustigmatophyceae.
- To review the factors responsible for the lipid content and composition changes and how lipid biosynthetic pathways work in the Eustigmatophyceae.
- To summarize progress of genetic engineering techniques in the improvement of lipid yields.

2 General description of the class Eustigmatophyceae

The Eustigmatophyceae are a small microalgal division considered as a separate lineage in Stramenopila (Patterson, 1989; Adl et al., 2005), Heterokontophyta (van den Hoek et al., 1995), or Ochrophyta (Cavalier-Smith and Chao, 1996). Stramenopiles are named for the strawlike hairs on the flagellar body. Heterokontophyta is obviously term describing two different types of flagella commonly present in the organisms belonging to it. And finally, Ochrophyta takes account of the ochre colour of the group. Flagellated cells possess a long forward-directed tinsel (hairy) flagellum and a shorter backward-directed smooth flagellum. The tinsel flagellum has two rows of tripartite tubular hairs known as mastigonemes, which are composed of glycoproteins (Ott and Oldham-Ott, 2003; Prior et al., 2009).

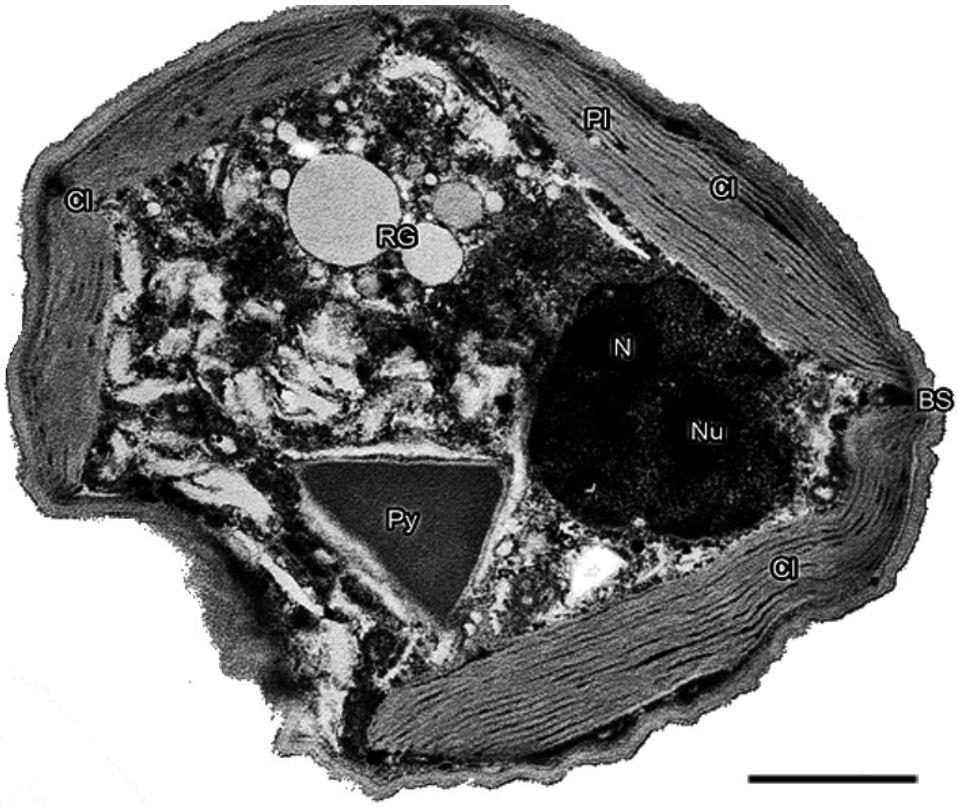

Fig. 1. Microphotograph from transmission electron microscopy (TEM) showing the strain E4f cell´s cross-section, depicting ultrastructure features of Eustigmatophyceae: Cl – chloroplast (without girdle lamella in this strain), N – nucleus, Nu – nucleolus, Pl – plastogluboli, Py – stalked polygonal pyrenoid, RG – reddish globule, the scale bar represents 1 µm, adapted from Fišerová (2012).

Long considered members of the Tribophyceae (Xanthophyceae), the algae now known as the eustigmatophytes were moved to their own class when Hibberd and Leedale (1971, 1972) discovered that these organisms differed from tribophytes structurally and in their pigment composition. Zoospores have a stigma (an eyespot) outside of the plastid (hence the name) and have one or two flagella. However, zoospores of the genus *Pseudotetraedriella* and *Pseudostaurastrum* are missing the stigma, Schnepf et al. (1995) observed that they are motile for only a couple of minutes and therefore they probably do not need the stigma. The eustigmatophytes have chlorophyll *a*, lacking chlorophyll *c*, and main accessory pigments there are violaxanthin and vaucheriaxanthin (Ott and Oldham-Ott, 2003; van den Hoek et al., 1995). The plastids of vegetative cells lack a girdle lamella

(peripheral stacks of three thylakoids). A stalked polyhedral pyrenoid is present in most eustigmatophytes except for the zoospores (Hibberd, 1981; Ott and Oldham-Ott, 2003; van den Hoek et al., 1995). Another distinctive structure in the cell is a reddish globule (Fig. 1, Fig. 2), present mainly in senescent (mature) cells; its name refers to the orange-red colour caused by carotenoids (Suda et al., 2002). Lamellate vesicles are the only characteristic ultrastructural feature in eustigmatophytes and are present in all so far investigated strains, although the appearance of lamellate vesicles depends on the life cycle. Fast growing cells have the lamellate vesicles full of lamellae, whereas the cells in stationary phase accumulate the storage polyglucans in them (Santos, 1990).

The number of species belonging to the class Eustigmatophyceae differs in various sources of information: Guiry and Guiry (2009) claim that the class consists of 5 families, 10 genera and 35 species in a single order, whereas according to Přibyl et al. (2012) the class comprises only over 20 species in 11 genera. In any case, all the members are unicellular algae with one or more yellow-green parietal plastids, living as a single cell or in colonies, thriving mostly in soil and freshwater habitats, probably their original environments. Only the genus of *Nannochloropsis* contains marine minute (2–4 μm) forms that live in the picoplankton (Eliáš a Neustupa in press; Procházková, 2012; Přibyl et al., 2012; van den Hoek et al., 1995). Moreover, one eustigmatophyte species (not yet precisely identified) acts as a symbiont in a freshwater sponge *Corvomeyenia everetti* (Frost et al. 1997). Undoubtedly, the eustigmatophytes are commonly found in various types of habitats although they are quite sparse, usually found in small numbers only. There are only a couple known exceptions. One is *Nannochloropsis limnetica* forming dense spring blooms (Fawley and Fawley, 2007) and the other one is *Trachydiscus minutus* growing nearly as a mono culture in cooling water of Temelín Nuclear Power Plant (Přibyl et al., 2012). In addition, Ghosh and Love (2011), analysed the diversity of microalgae in wastewater treatment plants in the USA and found out that according to *rbcL* marker, the eustigmatophytes dominated in one of them.

The eustigmatophytes are mostly of coccoid cell shape, e. g. *Eustigmatos*, *Nannochloropsis*, *Visheria*, *Chlorobotrys*), however, they also demonstrate a range of different cell morphologies, i.e. oval (*Pseudocharaciopsis* with a small stipe or attachment disk), ellipsoidal (*Ellipsoidion*), tetrahedral (*Pseudostaurastrum*) or irregular (*Visheria*), with their cell wall smooth or ornamented (*Vischeria*). The spherical species or nearly so are known from soil rather than from phytoplankton or tychoplankton samples. The yellow–green or greenish coccoid eustigmatophytes have often being mistaken for green algae (Ott and Oldham-Ott, 2003, Prior et al., 2009; Volkman et al., 1999). Vegetative cells have walls; in older cells more than one layer is usually visible, but their chemical composition is unclear at this time (Santos, 1990; Volkman et al., 1999a).

Reproduction of Eustigmatophyceae is by means of walled autospores (nonmotile spores without the potential to produce flagella) or naked (nonwalled) zoospores (flagellated asexual reproductive cells). Sexual reproduction has not been observed in eustigmatophyte algae (Ott and Oldham-Ott, 2003; van den Hoek et al., 1995). No flagellated stages are known for the genera *Monodopsis*, *Nannochloropsis*, and *Chlorobotrys* which do not produce zoospores (Neustupa and Němcová, 2001; Přibyl et al., 2012). Zoospores of other eustigmatophytes have one emergent flagel-

lum (*Eustigmatos*, *Pseudostaurastrum*, *Vischeria*), whereas those in the Pseudocharaciopsidaceae (*Ellipsoidion*, *Pseudocharaciopsis*) have two emergent flagella, both inserted near the apex of the cell (Ott and Oldham-Ott, 2003; van den Hoek et al., 1995). Přibyl et al. (2012) achieved induction of zoospore production in laboratory conditions by transferring the culture from light to dark conditions, and it was also temperature dependent.

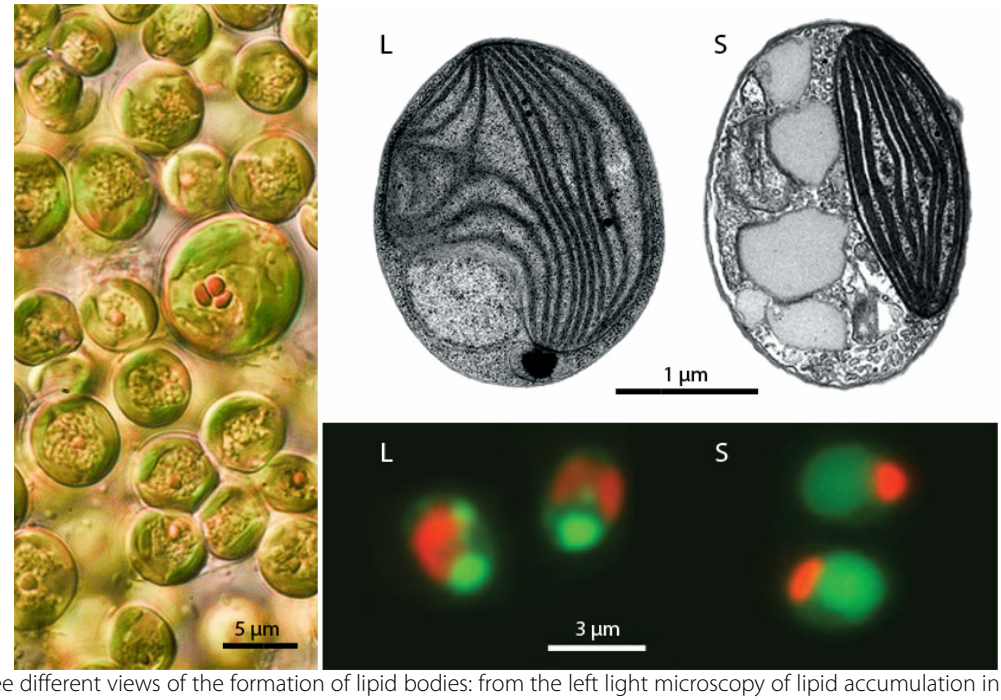

Fig. 2. Three different views of the formation of lipid bodies: from the left light microscopy of lipid accumulation in Bog D 9/21 T2d strain in stationary phase, reddish globules indicate senescent cells. TEM photographs of lipid droplets formation and the ultrastructural changes following N-starvation in *Nannochloropsis oceanica* (L – N-replete, S – N-depleted). The fluorescent microphotograph of lipid droplets in *N. gaditana* cells during logarithmic growth (L) on the left and during stationary phase (S) on the right: lipid droplets are fluorescently labeled with BODIPY (493/503) (green), chlorophyll autofluorescence (red); adapted from Fišerová, (2012); Radakovits et al. (2012); Vieler et al. (2012).

The monophyly of the Eustigmatophyceae was supported by the phylogenetic analyses of both *rbcL* and 18S rDNA genes (Andersen et al., 1998; Hegewald et al., 2007; Prior et al., 2009). Then, Eliaš et al. (unpublished) delineated more precisely phylogenetic relationships among the Eustigmatophyceae (Fig. 3) by performing phylogenetic analysis of *rbcL* and 18S rDNA genes. Two phylogenetically separated lineages have been revealed in the class Eustigmatophyceae, temporally designated Eustigmatales and Goniochloridales. More basal order of Goniochloridales comprised mostly of planktonic freshwater algae, formerly classified in Xanthophyceae, i.e. *Pseudostaurastrum limneticum*, *P. enorme*, *Goniochloris sculpta* and *Trachydiscus minutus*. The phylum Eustigmatales has been divided into three families: Monodopsidaceae, consisting of *Nannochloropsis*, *Monodopsis* and *Pseudotetraedriella*, Eustigmataceae including *Pseudocharaciopsis minuta*, *Ellipsoidion solitare* and *Eustigmatos*/*Visheria* complex (the two genera are identical; Procházková, 2012) and the remaining species of *Pseudellipsoidion edaphicum* and *Psedocharaciopsis ovalis* belonging to Pseudellipsoidionaceae (Eliáš et al., unpublished).

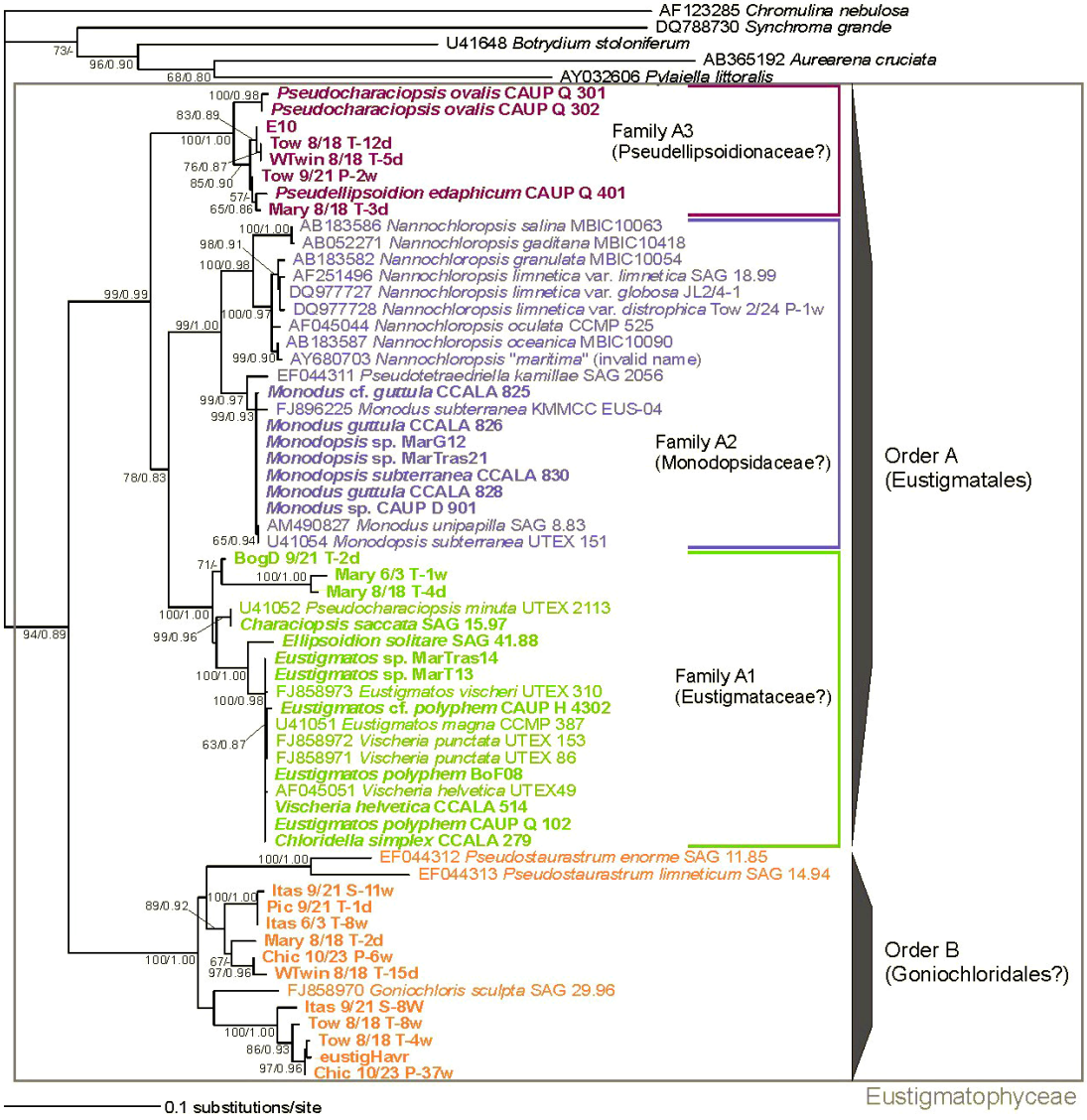

Fig. 3. Phylogenetic tree depicting relationships among the Eustigmatophyceae, rooted by *Chrysochromulina nebulosa*, based on maximum likelihood analysis of 18S rDNA using GTR+Γ+I model with bootstraps and aLRT values. As outgroups there is a small assembly of other stramenopiles. The new unpublished sequences are written in bold and the temporary names of genera and families are followed by question marks (Eliáš et al., unpublished).

3 Lipids in the Eustigmatophyceae

There are various types of lipids naturally occurring in the eustigmatophytes, such as sterols, mono-, di- and triglycerides, terpenoids and phospholipids (PL), as well as free fatty acids and others. Lipids are chemically and functionally very diverse; they are not defined according to their structure. Their common feature is hydrophobic interactions, because all of them are extractable by non-polar organic solvents (although some of them are amphipatic, as well). Lipids in general perform different biological functions, such as the structural unit of membranes (with capability of self-organization), means of energy storage and important signalling molecules. In this thesis the word lipid(s) is used in its *sensu stricto*, as a synonym for fats, as a subset of lipids called triglycerides (McMurry, 2007; Voet and Voet, 1995).

Fatty acids are present as a part of mono-, di- and triacylglycerols (TAG), galactolipids, phospholipids (PL) and they can also stand alone (Fig. 4). They are characterized by having a carboxyl group at one end of their aliphatic chain, and a methyl group on the opposite end. In the formulae for the FAs the first number gives the number of carbon atoms in FA molecule, the second digit indicates the number of double bonds, ω indicates the position of the first double bond in relation to the methyl end of the FA molecule. The ω-3 fatty acids belong to a series of polyunsaturated fatty acids characterized by the first double bond at the third position from the methyl end of the molecule (Fig. 4). The parent fatty acid in this family is α-linolenic acid (18:3ω-3) which can be converted by all herbivores, and probably all omnivores, to EPA and DHA by elongation and desaturation, but it is only poorly converted to other PUFA (Brett and Müller-Navara, 1997; Nordøy, 1991).

The interest of biochemical contents of the eustigmatophytes emerged in 1970s and first report of the lipids, largely sterols in a species of *Monodopsis subterranea* – in that case considered as a member of Xanthophyceae, came from Mercer et al. (1974). Twenty five years later Volkman et al. (1999b) presented the second report on the lipids in three freshwater eustigmatophytes *Vischeria punctata*, *Vischeria helvetica* and *Eustigmatos vischeri* in comparison with the lipid distributions of marine *Nannochloropsis* (*N. oculata*, *N. granulata* and *N. salina*) examined by the same authors before (Gelin et al., 1997; Volkman et al., 1993 and references therein). In 1980s the lipid research was concentrated mainly on the marine genus *Nannochloropsis* (Boussiba et al., 1987, Sukenik et al., 1989). A few other species were biochemically characterized, e.g. *Eustigmatos* cf. *polyphem* (Li et al., 2012; Zhang et al., 2012), *Trachydiscus minutus* (Iliev et al, 2008; 2010; Gigova et al., 2012; Petkov, 2012; Řezanka et al., 2011) and *Monodopsis subterranea* (Cohen 1994, 1999; Khozin-Goldberg and Cohen, 2006 ; Lie et Lin, 2005 ; Qiang et al., 1997). In 2011 Lang et al. performed a huge screening of 2 076 microalgal strains from the culture collection of algae of Göttingen University (SAG) and among them there were mentioned also the FA profiles of 17 eustigmatophytes and other 5 eustigmatophyte species which were previously classified in Xanthophyceae. The FA profiles are quite different from other reports (Table 1, 2); all of them were carried out on microalgae in the stationary phase of growth.

Qualitative and quantitative composition of lipids in microalgal cells is not a steady parameter. Since lipids perform different physiological roles in the cell, they follow dynamics of changing strategies of adaptations to the environment. The rate of synthesis of either storage neutral lipids, such as triacylglycerols (TAG), or structural polar lipids, such as monogalactosyl diacylglycerols (MGDG, Fig. 4), differs depending on the growth conditions and the growth stage (see below; Olofsson et al., 2012). In the Eustigmatophyceae TAG are mainly composed of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), such as palmitic (16:0) and palmitoleic (16:1ω-7) acid, although they are also formed by PUFA. Unlike plant or fish oil, TAG of the eustigmatophytes may contain only one kind of PUFA, such as EPA, ARA or linolenic acids (LA), in high quantities of them in the cell (Řezanka et al., 2011). MGDG is typically enriched with EPA and digalactosyl diacylglycerol (DGDG) is almost exclusively made of two types of molecular species, 16/16 and 20/16; sulphoquinovosyl diacylglycerol (SQDG) is the most saturated glycolipid containing mainly palmitic acid. 16:0, 16:1 and EPA quite often constitute over 75 % of the total fatty acids. The glycerolipids – MGDG and DGDG represent about 45 % of polar lipid classes in *Nannochloropsis*. Last but not least is diacylglycerol trimethyl homoserine (DGTS) – a specific lipid in algae. Phosphatidylcholine (PC) is dominated by monounsaturated fatty acids (MUFA), such as palmitoleic (16:1ω-7), oleic (18:1ω-9) and linoleic (C18:2ω-6) acids. Phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) are composed of relatively high percentage of arachidonic acid (ARA, 20:4ω-6; Cohen, 1999; Khozin-Goldberg et al., 2002; Řezanka et al., 2010; Schneider and Roessler, 1994; Sukenik, 1999; Sukenik et al., 1993).

However, it is worth mentioning that the same membrane properties could be achieved by different fatty acid proportions, even under the same growth conditions, such as temperature, light intensity and nutrition (Iliev et al., 2010). On the other hand, the composition of some other lipid classes, such as sterols, appears to be more stable. The eustigmatophytes contain simple sterol distribution dominated by either cholesterol (marine species) or 24-ethylcholesterol (freshwater species). Both groups contain long-chain alcohols and alkyl diols, as well as saturated and unsaturated monohydroxy fatty acids, although the carbon number ranges differ: C_{30} and C_{32} midchain hydroxy fatty acids are present only in marine species, whereas in freshwater *Vischeria* there are α- and β-hydroxy C_{24} to C_{30} acids (Patterson et al., 1994; Volkman et al., 1999a).

The abovementioned fatty acid distributions closely resemble between both the marine and freshwater eustigmatophytes but are quite distinct from those found in most other algal classes (Volkman et al., 1998, 1999a). In general, myristic (14:0) and linolenic (18:3) acids are much more abundant in marine microalgae than in freshwater ones, while oleic (16:1ω-7) and arachidonic (20:4ω-6) acids are more often found in freshwater microalgae (Liu and Lin, 2005). Moreover there are additional distinctive patterns in fatty acids distribution. For example, marine eustigmatophytes such as *Nannochloropsis* spp. contain EPA (20:5ω-3) but only small amounts of DHA (22:6ω-3), whereas haptophytes, such as *Pavlova* spp., contain both EPA and DHA in the similar proportion (Tonon et al., 2002). The attempt to detect DHA failed in *N. oculata*, *N. salina* and *Monodopsis subterranea* (Liu et Lin, 2005; Schneider et al., 1995; Tonon et al., 2002; Vazhappilly

and Chen, 1998; Volkman et al., 1993). However, other papers claim the presence of DHA in case of *N. oculata* and *N. limnetica*, *V. punctata*, *V. helvetica* and *E. visheri*, albeit in minor or trace amounts (Hu and Gao, 2003; Krienitz and Wirth, 2006; Roncarati et al., 2004; Vazhappilly and Chen, 1998; Volkman et al., 1999a). Similar distributions of FAs can also be found in some species of diatoms despite having much higher contents of the 14:0 and 18:4 fatty acids. Chlorophytes rarely contain significant amounts of EPA or DHA, but instead they have predominance of C_{18} PUFA such as LA (18:2ω-6) and ALA (18:3ω-3). Dinoflagellates have high levels of EPA and DHA and many of them contain the unusual fatty acid 18:5ω-3. However, in most marine organisms, fatty acids occur predominantly as polar lipids, such as glyco- and phospholipids, although levels of TAG can be high in microalgae grown under N-starvation (Volkman et al., 1999a).

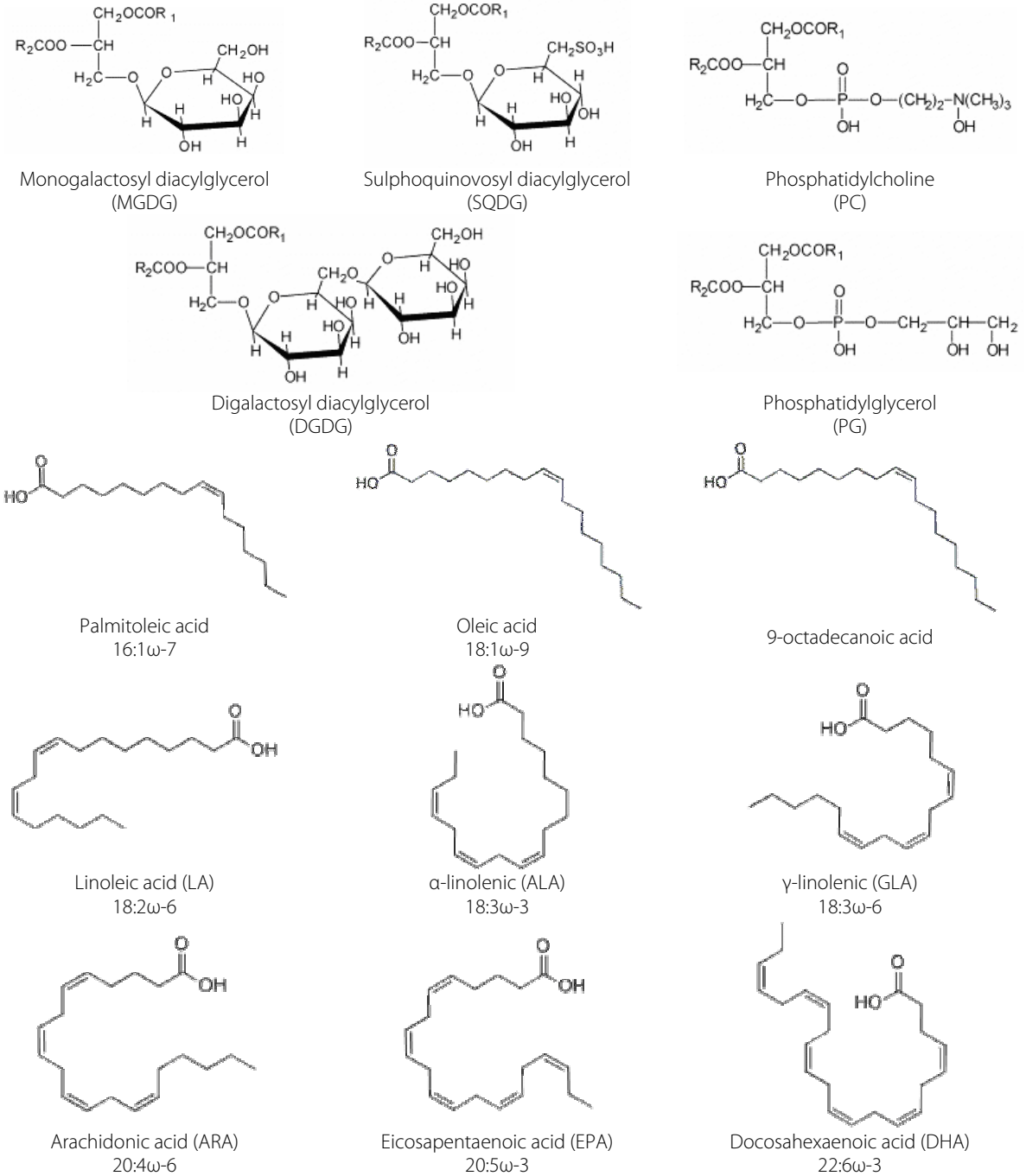

Fig. 4. Structures of the major polar lipid head groups, MUFA and PUFA in the Eustigmatophyceae.

FAs are considered a chemically relatively inert class of compounds that is easy to isolate from biological material and to be used as such as a chemotaxonomic marker to define groups of various taxonomic ranks. Lang et al. (2011) confirmed that lipid profiles of 2 076 microalgal strains from the culture collection of algae of Göttingen University (SAG) reflect phylogenetic relationships at the level of phyla and classes. Also, Gladu et al. (1995) suggested that the FA profile is a useful feature to identify the genus *Nannochloropsis*. In the field of algal chemotaxonomy, the similar attempt was made by Kumari et al. (2013), who employed FA signatures to differentiate macroalgae at higher taxonomic levels, such as family and orders. Generally, chemotaxonomy based on lipid profiles is successfully used in determining embryophytes (Mongrand et al., 2005) and also in prokaryotes, such as bacteria and more specifically cyanobacteria among which one can distinguish different species and strains (Shukla et al., 2012).

Most of nowadays´ works on FA profiles in the eustigmatophytes are mainly testing the optimal growth conditions, effects of nutrient starvation and the environmental conditions for the highest EPA yield. Unfortunately, there are lots of cross-effects of different variables which cause difficulties in optimization of microalgae cultivation. The productivity of EPA or any other FA is a function of four parameters – the EPA proportion in FAs, the FA content, the specific growth rate, and the biomass concentration at harvest. Specific growth rate is counted as the increase in cell mass per unit time (expressed in d^{-1}; Cohen, 1999; Hoffmann et al., 2010; Rocha et al., 2003; Řezanka et al., 2010).

Considering the scarcity and fragmentation of experimental studies in eustigmatophytes, it is quite problematic to sum and compare all the reported chemical analyses of lipid contents in the Eustigmatophyceae due to the great intraspecific variability caused by different cultivation conditions and methods of lipid extraction. However, an attempt to summarize nowadays knowledge of the lipid distribution in the Eustigmatophyceae was accomplished in the Table 1 and Table 2 with no ambition to include all of them. The summary of lipid contents in some representative eustigmatophytes is listed on the basis of their phylogeny affiliation (Fig. 3).

3.1 Diversity of fatty acids in the class Eustigmatophyceae

3.1.1 Eustigmatales – Eustigmataceae

3.1.1.1 The genus Eustigmatos/Visheria

Eustigmatos and *Vischeria* are forming together a single genus according to molecular phylogeny analyses of 18S rDNA and *rbcL* genes (Fig. 3; Procházková, 2012) and the overall similarity of the lipid distributions (Table 1) confirms the close taxonomic relationship among the species as well. In all three species – *Vischeria punctata*, *V. helvetica* and *Eustigmatos vischeri* the most abundant fatty acid is EPA, closely followed by both 16:0 and 16:1. Small amounts of C_{18} PUFA were also detected, e.g. LA. 14:0 and 15:0 were only minor constituents, and the DHA was only just detectable in trace amounts (Volkman et al., 1999a).

Moreover, additional lipid classes were investigated by Volkman et al. (1999b). Fig. 5 serves as an illustration of GC-MS result which is usually used in lipid distribution characterization.

Herein sterols, saturated and monounsaturated alcohols plus small amounts of saturated alkyl diols and sterols were detected. A series of saturated ∝- and β-hydroxy acids (i.e. 2- and 3-hydroxy monocarboxylic acids) is about 20 times less abundant than the total monocarboxylic FAs but comparable in abundancy to FAs of the same chain length. The function of the hydroxy FAs has not been satisfactorily explained yet, but they are supposed to figure in the formation of the lipid precursors of highly aliphatic biopolymers, e.g. algaenans, which are quite resistant to bacterial degradation and thus can survive into the sediment record, ultimately to become an important source of hydrocarbons in some crude oils (Volkman et al., 1999a).

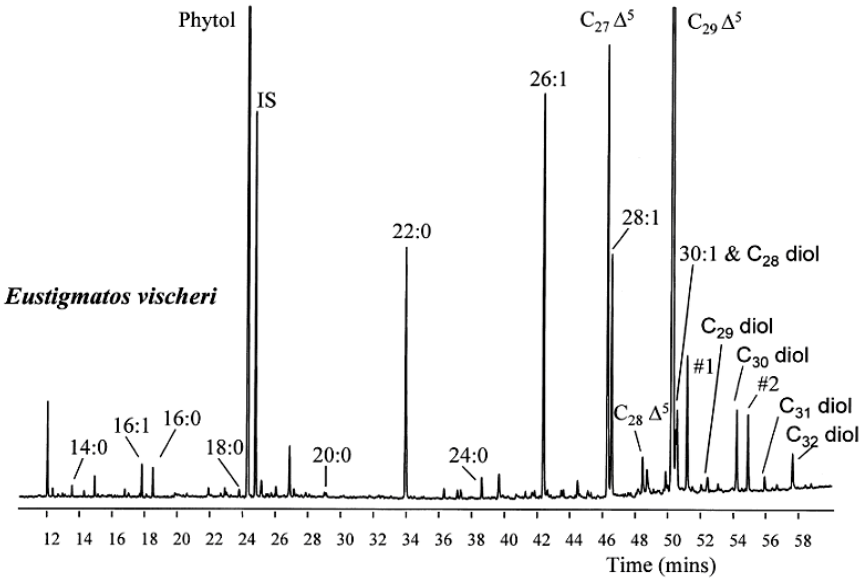

Fig. 5. Partial capillary gas chromatogram showing the distribution of alcohols, alkyl diols and sterols in *Eustigmatos vischeri*. Alcohols are designated x:y, where x is the chain length and y is the number of double bonds. The major alcohol present is phytol, which is presumed to originate from hydrolysis of chlorophyll *a*.The three sterols are cholesterol (labelled $C_{27}\Delta^5$), 24-methylcholesterol (labelled C_{28} Δ^5) and 24-ethylcholesterol (labelled $C_{29}\Delta^5$; this peak is off-scale by a factor of 4). Long-chain alkyl diols are denoted by C_x, where x is the number of carbon atoms. Unidentified compounds are indicated by #1 and #2; adapted from Volkman et al. (1999b).

Zhang et al. (2012) were the first to characterize the lipid accumulation in *Eustigmatos* cf. *polyphem*, which is quite high (TFA 60,6 % d.w.), as well as the lipid bodies formation under nitrogen limited conditions, growing it at 25 °C, with continuous illumination of 300 μmol m^{-2} s^{-1}.

3.1.1.2 Ellipsoidion sp.

Ellipsoidion sp. is not at the centre of interest on the field of biotechnologies but still there are few articles considering it a usable species and thus investigating the optimal growth conditions. The total lipid content of *Elliposidion* sp. ranged from 31 % to 36 % d.w. (EPA 18,8 % TFA) and the main fatty acids were similar to other eustigmatophytes (Table 1). The optimal conditions for EPA production by *Elliposidion* sp. are 25 °C, illumination of 145 μmol m^{-2} s^{-1} and pH 7,5, although the highest growth rate and total lipid content were achieved at pH 8,5 (Xuecheng, 2001).

3.1.2 Eustigmatales – Monodopsidaceae

3.1.2.1 Monodopsis subterranea

M. subterranea revealed the dominance of EPA (mainly in galactolipids) followed in decreasing order by 16:1ω-7 and 16:0 (in neutral lipids and phospholipids; Cohen, 1994, Qiang et al., 1997).

This particular species was found out to provide the highest EPA proportion (4,4 % d.w.), EPA content (49,3 % TFA), and EPA yield (1 g l^{-1}) in comparison to the other algae strains by Vazhappilly and Chen (1998) and Liu and Lin (2005). According to Cohen (1999) and Liu and Lin (2005) EPA productivity (mg l^{-1} d^{-1}) is maximal at 25 °C which corresponds to the optimal growth temperature although the highest proportion of EPA was obtained from 15 to 20 °C.

Table 1. Fatty acid distribution in six speices of the Eustigmatophyceae expressed as % (w/w) in total fatty acids. The ranging values are collected from several articles (see references) except for *Ellipsoidion* sp. Every strain was cultivated under phototrophic conditions in continuous illumination or 12:12 light-dark cycle at light intensity ranging from 50–180 µmol m^{-2} s^{-1}, at the temperature from 15 to 30 °C, in one of five standardized media: BBM, BG-11, modified Bourrelly, modified Provasoli medium and Zehnder liquid medium; with aeration ± enriched by CO_2. Algae were harvested in late exponential or stationary stage. * - DHA detected in trace amounts, # - unidentified isomery.

Species	***Monodopsis subterranea*** SAG 848-1 UTEX 151	***Vischeria punctata*** CS142 (UTEX 153) SAG 887-1	***Vischeria helvetica*** CS143 (UTEX 49) SAG 876-1	***Eustigmatos vischeri*** CS144 (UTEX 310) SAG 860-1	***Ellipsoidion sp.*** 70-01	***Trachydiscus minutus***
14:0	3,3	1–10	1,5	3,9	2,64	26–28
15:0		0,9	1	1,3		
15:1ω-5	20,6		4,3			
16:0	17,9–20,2	8,6–21,8	8,8–19,7	11,1–17,2	31,3	9–11,6
16:1ω-7	10,1–26,9	20,1–33,9	22,8–27,9	20,6–28,8	31,6 #	5–11,7
18:0	0,6–0,9	0,5	0,4	0,4	1,4	0,4–0,6
18:1ω-9	2,3–6,7	4,2–5,2	3,6–3,8	1,5	5,26	3
18:1ω-7		1,8	1,2	1	1,21	
18:2ω-6	0,4–2,4	8,6	6,5	5,2		5.7
18:3ω-6 **GLA**	0,4–1					0,2–5,9
18:3 ω-3 **ALA**	1,7–7,8	1,8	5,8–6,8	2,1	0,72	2,5
20:4ω-6 **ARA**	1,5–13,6		3,7		1	1,7–6
20:5ω-3 **EPA**	27,6–49,3	15,8–37,2	18,6–35,3	20,5–43,2	9,1	29–42
22:6ω-3 **DHA**		*	*	*		
9-Octadecanamid	16,3	31,8	27,2	39,6		
Other FAs:	d, h, i	e, g	e, g	e, g	b, c	a, f
References:	1, 3, 4 , 6, 8	3, 9	3, 9	3, 9	10	2, 5, 7

Other FAs: a - 12:0, **b** - 14:1, **c** - 16:1 ω-5, **d** - 16:2, **e** - 16:2 ω-4, **f** - 16:2 ω-6, **g** - 16:3 ω-3, **i** - 20:1ω-9, **j** - 20:2 ω-6, **k** - 20:3, **l** - 20:3 ω-3, **m** - 20:3 ω-6

References: 1 – Cohen, 1994; **2** – Iliev et al., 2010; **3** – Lang et al., 2011; **4** – Liu and Lin, 2005; **5** – Lukavský et al., 2010; **6** – Qiang et al., 1997; **7** – Řezanska et al., 2011; **8** – Vazhappilly and Chen, 1998; **9** – Volkman et al., 1999a ; **10** – Xu et al., 2001.

Cultivation under low light intensity or high biomass concentration enhanced the proportion of EPA (4,4 % d.w.). The highest EPA content is acquired towards the end of the exponential phase of growth, for although the growth slows down at this stage, biomass productivity is at its

highest (25,7 mg l^{-1} d^{-1} – the highest biomass productivity indoors), and EPA content is close to its maximum (Cohen, 1994, 1999). Qiang et al. (1997) introducing another cultivation design (of a flat plate reactor with a narrow light-path and intensive stirring which facilitates high cell concentration) unlike the Cohen´s semicontinous cultivation. Qiang then acquired two-fold higher maximal EPA productivity (58,9 mg 1^{-1} day^{-1}). The highest EPA cell content (32 % TFA, 3,8 % d.w.) occurred at the optimal density of 4 g l^{-1}, i.e. the density that yields the highest output rate of biomass per culture volume or area in any microalga ever reported. When algal density was maintained at its optimum, the proportion of EPA was highest, whereas the proportions of all other fatty acids except for 16:1ω-7 were at their lowest. High-concentration cultures would not only sustain a much higher volumetric productivity of biomass, but should also reduce operating costs. Thus, *M subterranea* seems to be a very promising species – providing high EPA percentage and FA content at a relatively high biomass concentration (Cohen, 1999; Liu and Lin, 2005).

3.1.2.2 The genus Nannochloropsis

Members of the genus *Nannochloropsis* are widely spread picoplankton mostly known from saline habitats. Until now, five different marine species (Fig. 3) have been recognized: *N. gaditana*, *N. granulata*, *N. oceanica*, *N. oculata*, *N. salina*; while the only one freshwater species – *N. limnetica* – has been described (Krienitz and Wirth, 2006; Krienitz et al., 2000). The species description for *N.* "*maritima*" is not known (Fawley and Fawley, 2007). Although their morphologies are so similar that they may be more likely distinguished on ultrastructural or molecular basis, comparison of the gene repertoires between *N. oceanica* and *N. gaditana* (Fig. 2) has indicated that the differences between these two species are comparable in magnitude to those observed between monocotyledonous and dicotyledonous plant species, which diverged from each other 150–200 million years ago (Vieler et al., 2012). Altogether, most of them have been confirmed as a promising source of FAs for aquacultures or biofuels production (Doan et al., 2011; Ferreira et al., 2009).

The lipid contents and FA profiles are very similar to other eustigmatophytes, with the exception of 16:1, which is according to the Table 1 and Table 2 present in both isomers 16:1ω-9 and 16:1ω-7 only in *Nannochloropsis* species, while the other eustigmatophytes possess only 16:1ω-7. Similarly, the FA composition of *N. limnetica*, a freshwater species, resembles to that of the marine ones, despite containing a slightly higher amounts of 14:0 and 16:1. In marine *Nannochloropsis* the proportion of short-chain vs. long-chain fatty acid content changes during the logarithmic and stationary phase of growth in the favour of short-chain SFA. However, in *N. limnetica* the PUFA concentration was higher in the stationary phase of growth and considerably higher in non-aerated cultures (Krienitz and Wirth, 2006; Roncarati et al., 2004; Tonon et al., 2002).

Although *Nannochloropsis* species have broad environmental tolerance, they usually require pH around 8 and temperature from 20 to 30 °C. Sukenik et al. (1989) attained the highest proportion of EPA (44 % TFA) in *N. oculata* by cultivation at suboptimal 20 °C with depressed growth rate, and low EPA productivity (Cohen, 1999). Surprisingly, in *N. salina* an exceptional result has been reported – the highest TFA and EPA productivity revealed at suboptimal temperature and high nitrate concentration (17 °C, 1 800 μmol l^{-1}), although the highest specific growth rate was

achieved at 26°C, the yield of TFA was four-fold lower than in the former conditions considered to be the most productive obtaining 3,5 % EPA d.w. (Hoffmann et al., 2010). Other reports often claim the opposite that the maximal productivities are performed in conditions when the growth rate is at its optimum (Liu and Lin, 2005).

Table 2. Fatty acid distribution in five *Nannochloropsis* speices expressed as % (w/w) in total fatty acids. The ranging values are collected from several articles (see references, if there is only one number, only one value is reported), every strain was cultivated under phototrophic conditions in continuous illumination or 12:12 light-dark cycle at light intensity ranging from 70–240 μmol m^{-2} s^{-1}, at the temperature from 17 to 26 °C, in one of standardized media: modified Bourrelly, f/2, 8-fold enriched f/2 and modified Fábrefas medium; with aeration ± enriched by CO_2. Algae were harvested in late exponential or stationary stage. # - unidentified isomery.

Species	***N. oculata*** CCAP 849/1 CS-179, CS-216, SAG 38.85, UTEX LB 2164	***N. salina*** CS-190 SAG 40.85	***N. oceanica*** NIVA-2/03	***N. gaditana*** SAG 12.99	***N. limnetica*** SAG 18.99
14:0	3–6,1	3,5–5	7,78–16,9	6,9	0,9–7,8
15:0	0,4–0,5	0,5			0,9
16:0	8,5–35,9	10,7–42,2	17,2–35,64	14,8–23,9	20,2–36,3
16:1ω-9	0,1–38,1	0,1	26,37–27,55		35
16:1ω-7	13–29,4	18,6–35,2	18,2 #	23,9–29,3	20,7–31,6
18:0	0,9–22,4	34,5	1,8–8,06		0,7
18:1ω-9	6,3–11,7	2,6–8,3	4,1–14,68	3,6–5,1	0,7–10,2
18:1ω-7	0,3–0,9	0,2		0,3	0,4–11,6
18:2ω-6	2,9–10	0,5–1,5	9,7	2,9	1,5–4,1
18:3ω-6 **GLA**	0,3–14,1	0,4–1,5	0,2–0,5		0,3
18:3ω-3 **ALA**	0,1–4,2	0,2–4,2		8,9	0,25
20:4ω-6 **ARA**	5,1–8,8	1,8–4	2,2–3,7	3,8–6,9	1–6,9
20:5ω-3 **EPA**	5,5–28,8	8,8–17,4	6,0–23,4	27,1–34,1	6,8–27,1
22:6ω-3 **DHA**	0–7,1				0,06
9-Octade-canamid	58	38,2		3,1	0,4
Other FAs:	d, f, g, h, m, n	b, d, f, g, h, m, n	a, i, k		c, e, i, l
References:	5, 7, 8, 9,	2, 5, 9	6, 10	1, 5	3, 4, 5

Other FAs: a – 12:0, **b** – 14:1, **c** – 15:1ω-5, **d** – 16:1 ω-3, **e** – 16:1 ω-5, **f** – 16:1 ω-13, **g** – 17:0, **h** – 20:3 ω-6, **i** – 20:1ω-9, **j** – 20:2 ω-6, **k** – 20:3, **l** – 20:3 ω-3, **m** – 20:3 ω-6, **n** – 20:4 ω-3.
References: 1 – Ferreira et al., 2009; **2** – Hoffmann et al., 2010; **3** – Krienitz and Wirth, 2006; **4** – Krienitz et al., 2000; **5** – Lang et al., 2011; **6** – Patil et al., 2007; **7** – Tonon et al., 2002; **8** – Vazhappilly and Chen, 1998; **9** – Volkman, 1993, **10** – Xiao et al., 2013

There is no evidence of photoinhibition observed (Rocha et al., 2003). The maximal growth rate (1,3 d^{-1}) in *N. salina* was obtained at 23 °C and 250 μmol m^{-2} $s^{-}1$ (Van Wagenen et al., 2012). Nevertheless, in other articles different values are always provided, e.g. in *Nannochloropsis* sp. an

optimal illumination 700 μmol m^{-2} s^{-1} was confirmed (Pal et al., 2011), whereas in *N. oceanica* the maximum daily production (0,7 g l^{-1} d^{-1}) at 1 030 μmol m^{-2} $s^{-}1$ and 24 °C (Sandnes et al., 2005). More complex insight was brought by Radakovits et al. (2012) taking account of culture density. Optimal lipid yields were acquired in *N. gaditana* with a starting culture density of ~3,6 g l^{-1}. Low-density cultures (<0,5 g l^{-1}) could have their growth inhibited by high light (>200 μmol m^{-2} s^{-1}); the higher density cultures (3–10 g l^{-1}) had good production between above 1 000 μmol m^{-2} s^{-1}, but without significant productivity raise on increasing the light from 1 000 to 2 000 μmol m^{-2} s^{-1} (sunlight intensity at midday). Self shading probably became the limiting factor at these densities (Radakovits et al., 2012, 2010).

3.1.3 Goniochloridales

3.1.3.1 Trachydiscus minutus

T. minutus produces lipids in a high amount (10–36 % d.w.) with considerable EPA content (22–42 %) and high EPA productivity of 88 mg l^{-1} d^{-1}, unusual high content of myristic (14:0) acid (about 26–54 %; Table 1, not all data shown), which is not so abundant in other algae; and quite distinct amount of GLA (11 %) also emerged. Among microalgae, *T. minutus* is nearly a top producer of EPA (Iliev et al., 2008; 2010; Lukavský et al., 2010; Řezanka et al., 2010).

The optimal growth temperature in *T. minutus* according to different studies is about 26 °C (tolerance from 20 to 32 °C, lethality above 42 °C; growth inhibition below 15 °C; Gigonova et al., 2012 ; Iliev et al., 2010 ; Lukavský et al., 2010). The percentage of lipids increased significantly at low temperatures (from 21,8 % to 35,2 % d.w.), regardless of light intensity, reaching the highest value at 15 °C at the expense of carbohydrates, while protein content remained quite steady (34±4 %), as the main structural and metabolic component of the cell (Gigova et al., 2012). The changes in growth rate and percentage of PUFA proved that *T. minutus* requires lower light intensity (mainly in low culture densities light intensity should not exceed 160 μmol m^{-2} s^{-1}). Iliev et al. (2010) do not sufficiently discuss the particular effect of light although in high light intensities (at the same temperature) the lipid distribution is dramatically changed with minor contents of EPA and 14:0 unlike the predominant 16:0 and LA.

4 Environmental effects on the lipid distribution changes

The lipid content, as well as the overall chemical composition of microalgae can be manipulated and even dramatically changed by varying growth conditions (Shifrin and Chisholm, 1981). Under optimal growth conditions, cells mainly synthesize proteins that are required for growth. In suboptimal, conditions the cellular composition is changed due to the accumulation of lipids, carbohydrates and other metabolites (Mata et al., 2010; Sukenik, 1999).

Apart from that there are also changes in the cell contents associated with the life span. The main trend in growing algae follows a decrease of the main PUFA in accumulated lipids over the stationary phase concomitantly with an increase in the short-chain fatty acids (Liu and Lin, 2005; Roncarati et al., 2004). Nevertheless, the influence of various factors (either exogenous or endogenous) on microalgal growth is linked into intertwined cross effects that seem to be very complex and difficult to explain in detail.

Fatty acids occur in various lipids having different physiological functions. Although the total lipid content may increase under specific conditions, it usually occurs concomitantly with disproportional variations in fatty acid composition, which depend on light intensity and quality, photoperiod, temperature, salinity, pH and most importantly nutrient availability (e.g. compounds of nitrogen, phosphorus and sulphur). Less is known about effects on other lipid classes (Liu and Lin, 2005; Shifrin and Chisholm, 1981; Sukenik, 1991; Volkman et al., 1998). The impact of various growth conditions and nutrition limitation or even deficiency (e.g. physiological forcing) on the FA productivity have been studied mainly in *Nannochloropsis* (Converti et al., 2009; Hu and Gao, 2003; Lin et al., 2012; Pal et al., 2011; Recht et al., 2012; Simionato et al., 2011; Sukenik and Carmeli, 1990), and also in *Monodopsis subterranea* (Khozin-Goldberg and Cohen, 2006), *Trachydiscus minutus* (Řezanka et al., 2010, 2011) or *Ellipsoidion* sp. (Xu et al., 2001; Xuecheng, 2001).

4.1 Nutritional factors

4.1.1 Carbon supplement

The elevation of carbon dioxide supply causes an increased content of long-chain FAs, mainly the ω-3 and ω-6 PUFA, while raising the ω-3/ω-6 ratio, but almost no differences in EPA levels. CO_2 enrichment also accelerates the growth of the culture. Otherwise an increased proportion of short-chain fatty acids and a decrease in long-chain fatty acids occur when low CO_2 concentrations are provided (Hu and Gao, 2003; Roncarati et al., 2004).

The issue of aeration is intimately connected to the effective CO_2 supplementation but again it is not achieved without obstacles. Comparison of aerated (fast growing) versus nonaerated (slower growing) cultures of N. limnetica showed a remarkably higher FA content in the non-aerated cultures (Krienitz and Wirth, 2006). The impact of gas sparging to N. salina growth with both ambient air and CO2-enriched air in bubble column photobioreactors was examined. The optimal gas-to-culture volume 0,18 min–1 resulted to the lipid content of 60 % and specific growth rate 0,22 d^{-1} – both considerably high values (Arudchelvam and Nirmalakhandan, 2012a). The productivity was then raised by 50 % by sparging with 0,5% CO_2-enriched air along with nitrogen

starvation (Arudchelvam and Nirmalakhandan, 2012b). Moreover CO_2 enriched aeration helps to keep pH on a desired value. On the one hand, aeration is besides the CO_2 supply, is a convenient mixing device which is suspending both the nutrients in medium and the cells themselves preventing them from sedimentation. On the other hand, there is a serious threat associated with shearing forces and its negative effect on the growth rate. Therefore, Rocha et al. (2003) recommended a small air flow rate with large bubbles as being more efficient for CO_2 mass transfer.

4.1.1.1 Mixotrophy vs. photoautotrophy

Nannochloropsis species can also use organic carbon sources (Rocha et al., 2003; Sandnes et al., 2005), although nitrogen assimilation along with the highest growth rate in *N. oculata* are observed in the presence of dissolved inorganic carbon rather than of dissolved organic carbon which is used mainly after depletion of the former (Lin et al., 2012).

In order to compare the photoautotrophic and mixotrophic growth conditions with enriched CO_2 aeration, Hu and Gao (2003) observed that the elevation of CO_2 in photoautotrophic culture enhanced growth and raised the production of both TFA and EPA in *Nannochloropsis* sp. Mixotrophic cultures growing on sodium acetate brought a greater protein content and less carbohydrates and lipids. This result is explained by Sforza et al. (2012) who claim that excess CO_2 stimulates photosynthesis but blocks the metabolism of organic substrate cancelling the advantages of mixotrophy which can be very helpful in light dark cycle to enhance lipid productivity during the night, but this happens only if CO_2 is not supplied (Sforza et al., 2012).

Mixotrophy can be also exploited in the two phase microalgae growth in open systems giving an opportunity to increase both the biomass and lipid content of culture at the later growth phase. The usage of glucose, sucrose and glycerol additional in mixotrophic growth led to higher biomass productivities (87, 103 and 100 mg l^{-1} d^{-1} respectively), compared to 27 mg l^{-1} d^{-1} for photoautotrophic control culture – all of them with the similar lipid contents except of glycerol showing higher FA content (Das et al., 2011). Another experiment with mixotrophic growth on glucose brought a similar result – the biomass yields are higher with not significantly changed FA profile, thus it is supposed to be better method to produce EPA (Xu et al., 2004). Experiments with mixotrophic and heterotrophic growth in *M. subterranea* were performed by Liu and Lin (2005) and Vazhappilly and Chen (1998), respectively. But the effects of both phenomena were not discussed.

4.1.2 Nitrogen concentration and source influence

The eustigmatophytes (unlike e.g. green algae) favour the synthesis of neutral lipids under stress limited growth. The nitrogen deprivation (e.g. in *Ellipsoidion* sp., *M. subterranea*, *Nannochloropsis* sp.) has been reported to enhance the FA content (up to 70 % d.w.), accompanied by a sharp decrease in proportion of PUFA with reduced the level of ARA as well as EPA, while 16:0 and 16:1 represented about 70% to 80% of TFA. In addition, 16:1 and C18:1ω-9 showed an increased percentage with decreasing nitrate concentration (Hoffmann et al., 2010; Hu and Gao, 2003; Khozin-Goldberg and Cohen, 2006; Rodolfi et al., 2009). *Vice versa*, the higher the nitrate concentration, the higher the EPA and PUFA contents of the alga. At the same time, the content of total

FA decreased (Xu et al., 2001). On the other hand it is a lot dissimilar to *T. minutus* in which the proportion of ARA exceeded 60 % TFA. This can be ascribed to the fact that, though structurally similar, EPA and ARA are biosynthesized by two different mechanisms (Řezanka et al., 2010).

The effect of different nitrogen sources on the growth rate and FA composition is also interesting. Nitrate-N is widely used as the sole nitrogen source for microalgae, because, compared to ammonium-N, it is less likely that a pH shift in the medium. The pH in ammonium-N containing culture tends to drop as a result of ammonium assimilation, while nitrate causes the pH to rise. It requires more energy from microlgae to assimilate the nitrate-N than that of ammonium-N, so they prefer to use ammonium-N from the medium. The alga grew poorly in N-free medium or medium with urea as the sole N source. This is corroborated by experiments resulting in higher specific growth rate and total lipid content (0,31 and 33,3 %, respectively) in ammonium containing medium than those in nitrate medium (0,29 and 27,6 % of TFA, respectively) of the same concentration (Xu et al., 2001).

TAG containing especially SFA and MUFA can efficiently generate more energy than carbohydrates upon oxidation, thus constituting the best reserve for rebuilding the cell after stress. Furthermore, the lipid synthesis possibly relaxes overreduced electron transport chain (ETC) in consequence of nitrogen starvation and photosynthesis operation. The lipid production thus serves as a protection of inner structures against the reactive oxygen species which are being formed in ETC. The effect of low temperatures seems to be quite similar (see below; Hoffmann et al., 2010; Rodolfi et al., 2009).

Genes that are most upregulated in *N. gaditana* during nitrogen deprivation are those involved in nitrogen assimilation and protein degradation or recycling and lipid biosynthesis, whereas many of the most downregulated genes are involved in photosynthesis and gluconeogenesis, which could help direct carbon flux away from carbohydrate biosynthesis into lipid biosynthesis. Because *N. gaditana* constitutively produces TAG even during logarithmic growth, a possible explanation for this low amount of differential transcript accumulation is that the lipid production machinery may already be abundant within the cell, and the existing levels can manage increased metabolic flux (Radakovits et al., 2012).

4.1.3 Phosphorus concentration influence

The signs of N- and P-starvation are alike in the eustigmatophytes, however, the P-replete conditions are effecting them differently. It was shown that high phosphate concentration led to a higher total content of all – SFA, MUFA and PUFA in *N. limnetica* (Krienitz and Wirth, 2006; Rezanka et al., 2010). Fig. 6 illustrates the proportional changes of FAs during P-starvation. The content of TAG was increased more than 5-fold (Fig. 6), similarly to N-starvation. The overall proportion of polar lipids decreased, especially MGDG and PL, whereas DGDG and DGTS were reduced only slightly. This resulted to an EPA percentage decrease at the expense of a substantial increase in 18:0, 18:1ω-9 and 20:3ω-6. DGDGs were found to be critical for the functional intactness of the thylakoid membranes as DGDGs support bilayer structures unlike MGDGs. The

MGDG/DGDG ratio is 2:1 in control culture, decreasing to about 1:1 under P-deprivation (Khozin-Goldberg and Cohen, 2006).

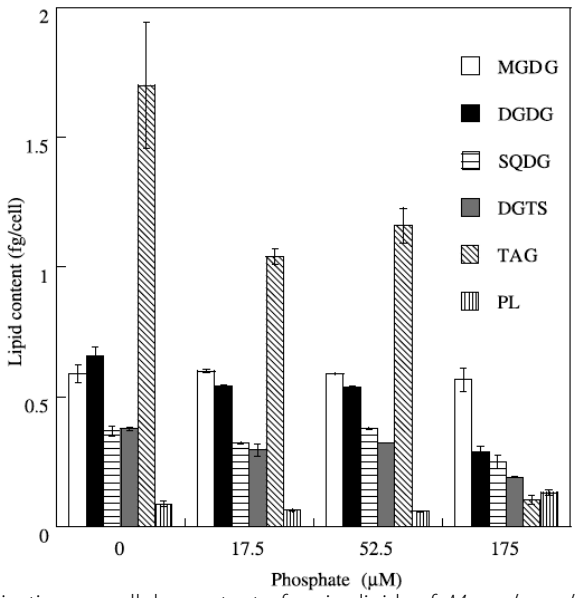

Fig. 6. Effect of phosphate deprivation on cellular content of major lipids of *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006).

4.2 Environmental factors

4.2.1 The effect of the light intensity

The modulation of lipid content and composition by light intensity has been demonstrated in *Nannochloropsis* sp. which shows a high lipid content under high light intensities, concomitant with an increase in the relative abundance of SFA and storage TAG in general, and with a reduction of PUFA (mainly a content of MGDG in thylakoid membrane) as compared to cells grown in light limiting conditions (Sukenik et al., 1989), when *Nannochloropsis* sp. preferentially synthesizes structural galactolipids enriched with EPA up to 40 % TFA; this variation coincided with an increase in the amount of thylakoid membranes (Sukenik and Cameli, 1990; Sukenik et al., 1993; Sukenik, 1999). Obviously, the rapid and very dynamic metabolism of FAs, similarly to other compounds, such as pigments and chlorophyll in particular, may be an adaptive response of cells to the changes of light intensities (Rocha et al., 2003).

The effects of a 12:12 h light-dark cycle on the lipid distribution in *Nannochloropsis* were examined in order to simulate more natural conditions. Variations in lipid biosynthesis rates were estimated by ^{14}C acetate incorporation into lipid classes and were attributed to the changes in the cellular metabolic state due to light availability. Radiolabeled intermediates provided exogenously to microalgae were taken up and converted to final PUFA products (Metz et al., 2001). Cellular content of TFA followed the diurnal variations in total lipids (Fig. 7); both TAG and MGDG gradually increased until a maximal rate was achieved 8 h after the lights were turned on whereas no lipogenesis was observed during the dark period (Sukenik et al. 1989; Sukenik and Carmeli, 1990). Lipids were preferentially consumed during the dark period because of the cell division and the respiration. This explained the fluctuation in 16:0 and EPA during the day, as TAG containing mostly 16:0 were produced during the light period and then they were used up accordingly to the

relative increase of EPA percentage. Alongside, no nitrogen was assimilated at night and no proteins were produced, although proteins and carbohydrates decreased only slightly (Fábregas et al., 2002; Sukenik et al. 1989; Sukenik and Carmeli, 1990).

One of the most important enzymes in fatty acid synthesis, acetyl-CoA carboxylase (ACCase), which catalyzes the formation of malonyl-CoA, is an ATP dependent enzyme, and its activity is enhanced by light. The low availability of ATP in the dark may decrease the ACCase activity and cause a reduction in malonyl-CoA pool. Other enzymes of the fatty acid synthesis (FAS) system are ATP and NADPH dependent, and the fact that low levels of malonyl-CoA induce early termination of fatty acid synthesis may explain the low synthesis rate of lipids in the dark (Schweizer, 2004; Sukenik and Carmeli, 1990). Nevertheless, excess of radiation can lead to the formation of dangerous reactive oxygen species and oxidative stress causing the process of photoinhibition, as photosystem II is continuously damaged. This complex must be therefore continuously repaired which is energetically demanding and may strongly influence the biomass productivity (Simionato et al., 2011).

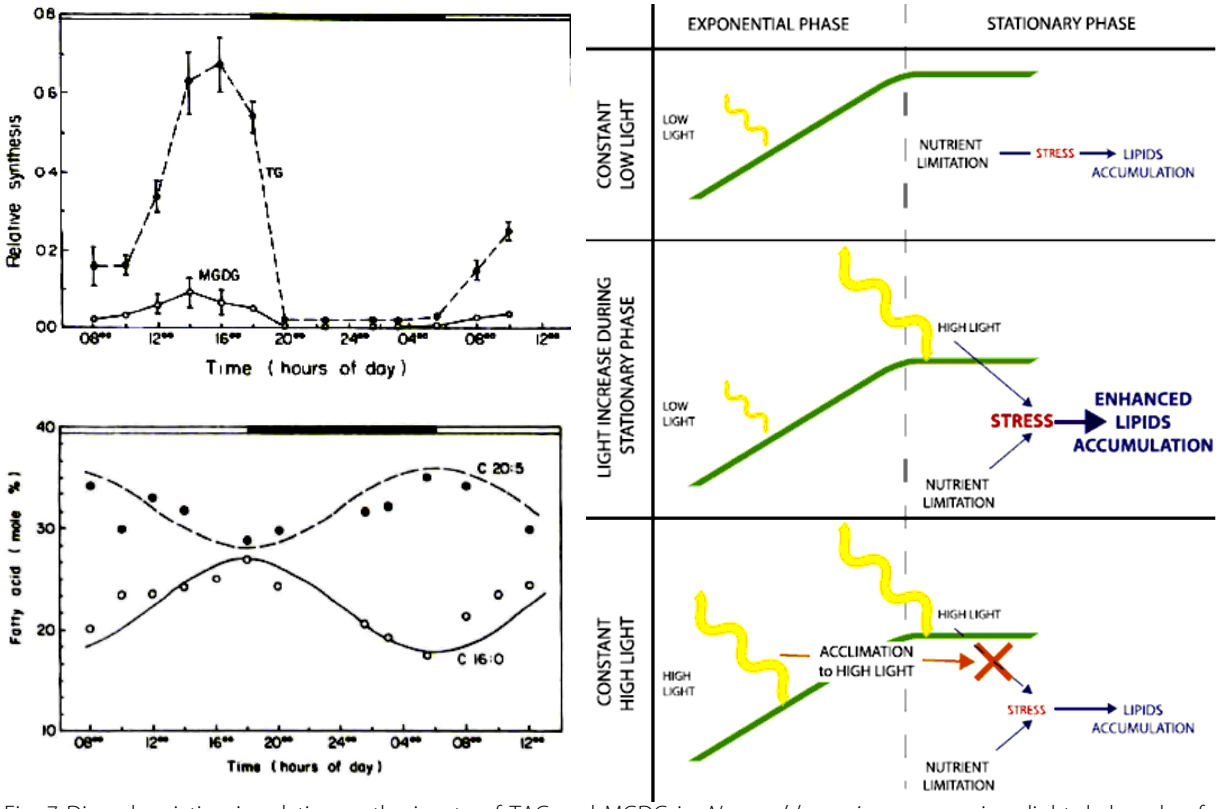

Fig. 7 Diurnal variation in relative synthesis rate of TAG and MGDG in *Nannochloropsis* sp. grown in a light-dark cycle of 12:12 h is shown on the left upper side and the diurnal changes in the mole percentage of the major FAs on the left lower side. On the right there is a scheme of light effect on lipids accumulation in *N. gaditana*: during the stationary phase, the stress due to nutrients deprivation induces lipids biosynthesis. A sudden increase of the illumination intensity during the stationary phase enhances nutrients deprivation and lipids accumulation. If cells are grown in strong light, activation of the acclimation response decreases the light induced stress on the cells, with no enhancement of lipids accumulation. Values represent mean ± SD obtained from three measurements of ^{14}C activity by scintillation counting. The black horizontal bar represents the dark period;.adapted from Simionato (2011) and Sukenik and Carmeli (1990).

Simionato et al. (2011) came up with a hypothesis that light intensity alone is not the major signal affecting lipids accumulation due to a missing direct regulatory link between the light stress and lipids accumulation. They observed that the growth kinetics in *N. gaditana* was not light limited but depended on other factors, like CO_2 and nutrient availability. Thus, these results suggest

that the alga has a remarkable capacity of acclimating to different photon fluxes by adapting its photosynthetic apparatus. Hence, considering the influence of light on the lipids productivity of *N. gaditana*, three different cases can be distinguished (Fig 7). In the first scenario, cells are exposed to a constant low or moderate light and the lipid accumulation is activated by nutrient limitation during the stationary phase. In the second case, cells grow at a moderate light intensity but are subjected to a light stress during the stationary phase. In this case, the irradiance changes in the phase where cells are prone to photoinhibition, fortifies nutrients deprivation stress and enhances lipids accumulation. The third case is the one of a constant exposition to a strong irradiance. In this case, cells are capable of acclimating to the high light intensity by enhancing protective mechanisms and optimizing their photosynthetic apparatus. Thanks to this response, in the stationary phase the light stress perceived is lower with small effect on lipid production, which is again induced by nutrients deprivation (Simionato et al., 2011).

4.2.2 Temperature

Converti et al. (2009) and Iliev et al. (2008, 2010) failed to find any clear correlation between the temperature and FA distribution changes. Hence, they concluded that the changes at different light intensities were much more expressed. On the other hand Gigova et al. (2012) claimed that the role of temperature was more pronounced than that of the applied light intensity in regulating growth of *T. minutus*. They also gave evidence of dramatically elevated activities of superoxide dismutase, catalase, and proteases at 40 °C, whereas at 15 °C they were suppressed, except for glutathione reductase. The effect of light was to enhance or decrease the temperature stress responses, depending on intensity, which is in agreement with Simionato et al. (2011; Gigova et al., 2012).

Hoffmann et al. (2010) brought an interesting insight into temperature induced physiological changes in *N. salina* compiling the knowledge of temperature-induced changes in FA composition mainly in cyanobacteria. In general, suboptimal temperature causes a decreased synthesis of SFA and increased concentrations of unsaturated FAs (Sukenik, 1993). This could have been provoked by a low temperature-induced decrease in the uptake of nitrogen, which is temperature sensitive. This would stop the protein synthesis also leading to the loss of chlorophyll *a*, which may results in an overreduced state of the ETC. Nevertheless, the cells counteract this problem by an enhanced accumulation of short-chain SFA and MUFA forming TAG – an analogical reaction occurring under nitrogen deprivation (described above). Low temperatures negatively affect the membrane fluidity and cells counteract by an increased synthesis of PUFA, e.g. EPA. Additionally, an enhanced EPA content in glycolipids of thylakoid membranes protects the photosynthetic apparatus against low temperatures. This is corroborated by the fact that photosystem II is very temperature sensitive. Therefore, the enhanced EPA concentration seems to be a protective mechanism of *N. salina* against low temperature conditions (Hoffmann et al., 2010). The trustworthy explanation of the high temperature effects is still missing in eustigmatophytes.

Biosynthesis

The knowledge of processes and pathways involved in the biosynthesis of FAs, especially EPA, is important for future genetic engineering utilization, as well as for modifying the growth conditions so as to attain higher EPA productivity. Because of the absence of appropriate molecular biology tools, discovering the biosynthesis of PUFA in microalgae has relied mostly on radiolabeling and inhibitor and mutants studies (Khozin-Goldberg et al., 2002; Schneider and Roessler, 1994; Schneider et al., 1995). Following proteomic analyses of purified lipid bodies, gene expression and transcriptome studies have discovered several key enzymes in TAG metabolism.

4.3 Fatty acid synthesis

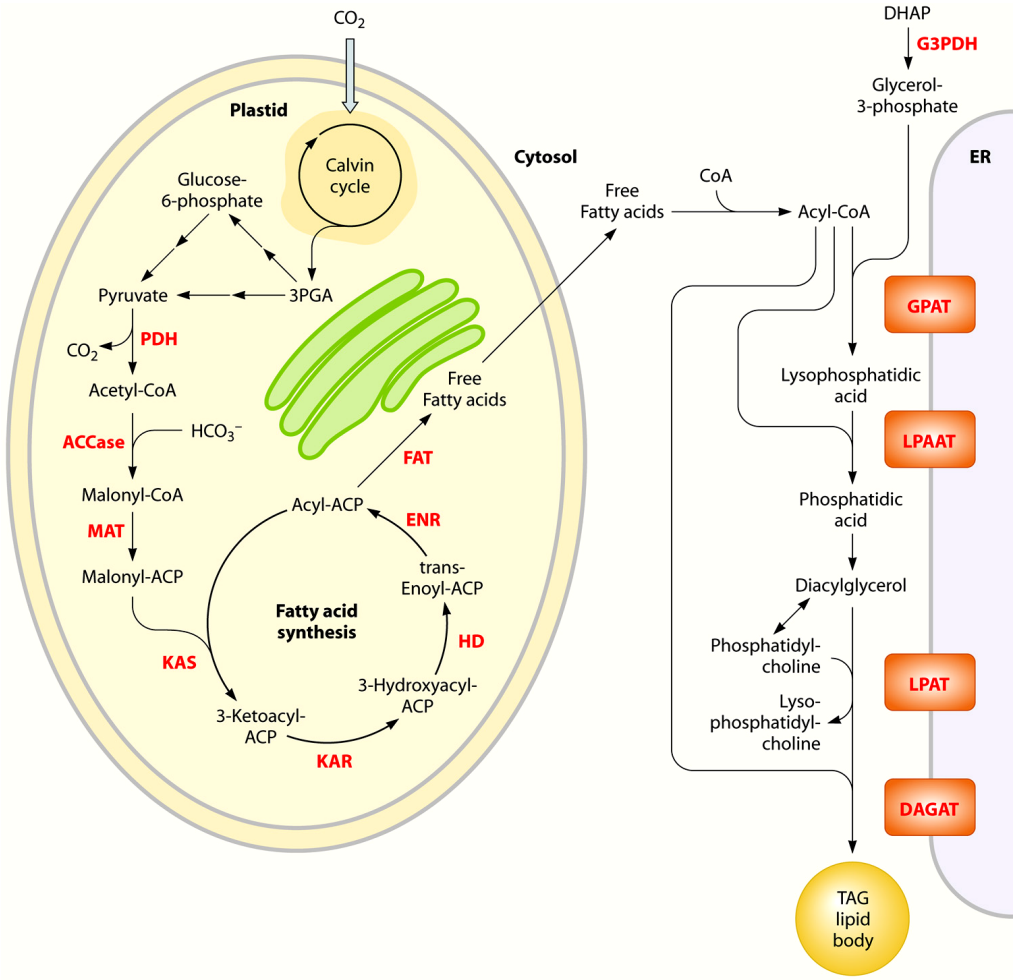

Fig. 8. Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes shown in red. Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, gycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerols; adapter from Radakovits et al. (2010).

Fatty acid biosynthesis is initiated by acetyltransferase, loading the acyl primer, usually acetate, from CoA to a specific binding site on FAS. Then elongation occurs by several iterative cycles. Each cycle includes malonyl-transacylation from CoA to the enzyme by malonyl transferase condensation of acyl-enzyme with enzyme-bound malonate to 3-ketoacyl-enzyme by 3-ketoacyl syn-

thase, reduction of the 3-keto- to the 3-hydroxyacyl intermediate by ketoacyl reductase, dehydration of 3-hydroxyacyl enzyme to 2,3-trans-enoate by dehydratase, and, finally, reduction of the enoate to the saturated acyl-enzyme by enoyl reductase. In the process of termination, the products are removed from FAS either by transesterification to an appropriate acceptor or by hydrolysis (by palmitoyl transferase and thioesterase). Fatty acid synthases (FAS) can be devided into two classes, type I and II. Type I systems occur as large multi-enzyme complexes in endoplasmic reticulum of eukaryotes whereas type II is typical for bacteria and organelles (chloroplasts/mitochondria) of plants, animals and algae (Schweizer, 2004; Vieler et al., 2012).

There are also polyketide synthases (PKSs) which carry out some of the same reactions as FAS, but it is often abbreviated in order to produce highly derivatized carbon chain, e.g. keto and hydroxy groups alongside with double bonds in the trans configuration. Such an enzyme may be therefore useful in the production of new families of antibiotics. FAS and PKS are able to produce long-chain SFA or MUFA. PUFA synthesis is much more complicated (Metz et al., 2001).

4.4 Triacylglycerol assembly

During the assembly of TAG, which accumulates in lipid droplets (Fig. 8), diacylglycerol acyltransferase (DAGAT) uses acyl-CoA as acyl donors in the final step of the third FA addition to DAG. Another TAG biosynthesis pathway is the acyl-CoA-independent transfer of a glycerolipid-bound fatty acid to DAG, which is present in most eukaryotes and is performed by a so called phospholipid:DAG acyltransferase (PDAT). These are the main targets of genetic and metabolic engineering in TAG assembly process (see below). In order to synthesize the rather simple set of fatty acids found, a minimal set of six desaturases and one elongase is required (Vieler et al., 2012; Zhang et al., 2012).

4.5 Polyunsaturated fatty acid synthesis

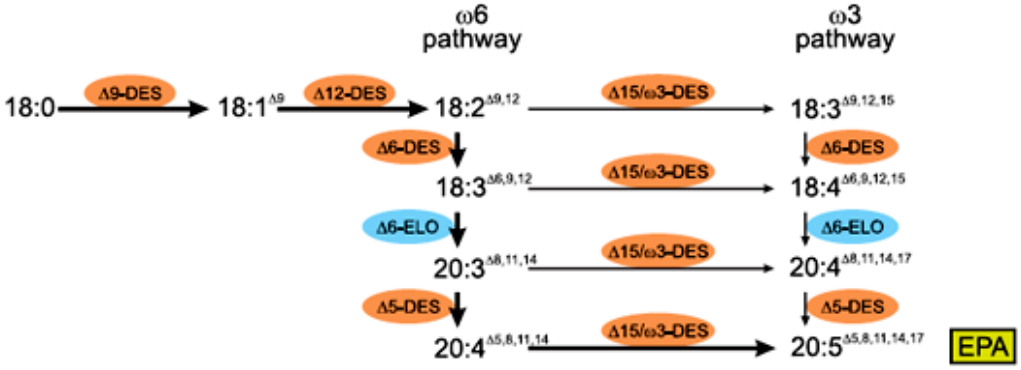

Fig. 9. Proposed pathway of desaturation (DES) and elongation (ELO) of fatty acyl chains in the endoplasmic reticulum of *Nannochloropsis;* adapted from Vieler et al. (2012).

Browse and Somerville (1991) described PUFA biosynthesis in higher plants as a complex process consisting of two different pathways: the prokaryotic and eukaryotic - without functional but only with structural difference observed. Khozin-Goldberg et al. (2002), investigating PUFA biosynthesis in a eustigmatophyte algae (Fig. 9), ascertained it was even much more complicated process than in plants. Unlike that of higher plants, algal PUFA may contain up to six double bonds with chain lengths as long as 22 carbon atoms, e.g. DHA synthesis from acyl-CoA requires

approximately 30 distinct enzymes and nearly 70 reactions, including the four repetitive steps of the FAS cycle (Metz et al., 2001). It is suggested that in the eustigmatophytes the ω-6 pathway may predominate (Fig. 9). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are both intermediates in the synthesis of 20:5ω-3. The C_{18} fatty acids are desaturated to 18:3ω-6 while attached to PC and then it is again elongated and desaturated from 20:3ω-6 to 20:5ω-3, while attached to PE or diacylglycerol trimethyl homoserine (DGTS) in either prokaryotic or eukaryotic pathways. Interestingly, in the monogalactosyl diacylglycerol (MGDG), the share of molecular species containing EPA in both the *sn*-1 and *sn*-2 positions (eukaryotic-like) increased at the expense of those containing FAs with shorter chain length at the *sn*-2 position (prokaryotic-like). Thus it is suggested that manipulation in favour of eukaryotic-like PUFA synthesis would be useful in order to increase EPA production. Since the majority of EPA is found inside the plastid esterified to MGDG, although EPA synthesis occurs exclusively outside of the plastid, this raises interesting questions about the lipid trafficking pathways and, taking into account the enrichment of EPA in DGTS as opposed to PC and PE (Cohen, 1999; Khozin-Goldberg et al., 2002; Schneider and Roessler, 1994; Sukenik, 1999, Vieler et al., 2012).

5 Genetic engeneering approaches

In case of the potential of genetic engineering in algae there are two widespread beliefs. One suggests that there is no apparent need to genetically modify microalgae as for it seems likely that among the thousands of microalgal species and billions of strains having colonized almost every photic niche on the earth, many organisms suitable for outdoor mass culture and biofuel production might be found. Moreover, there are fears of biological contamination, as well as restrictive legislation which still hinder the broader utilization of genetically modified organisms (Mata et al., 2010; Rodolfi et al., 2009). The opposite approach is promoting genetic engineering in microalgae as a powerful tool for improvement of their traits as an analogy to plant-growing, which has been through a long history of breeding that brought a lot of success. Genetic and metabolic engineering of microalgae could be used, e.g. to eliminate photosaturation and photoinhibition, which is expected to significantly increase productivity of outdoor cultures and greatly improve the economics of microalgae oil production. However, it will require long-term research and funding to get a new "green yeast" (Kilian et al., 2011; Rodolfi et al., 2009).

The first to appear was the molecular taxonomy interest in the Eustigmatophyceae focusing on 18S rDNA and *rbcL* gene sequences for phylogenetics and molecular taxonomic purposes (Andersen et al., 1998; Fawley and Fawley, 2007; Hegewald et al., 2007; Procházková, 2012; Yang et al., 2012). Several research groups have set out to sequence the genome of *Nannochloropsis gaditana* and *N. oceanica* which have recently become available, promoting both species to emerge as important model species for algal biofuel production. According to the molecular clock the two algae diverged 200 million years ago; both species are similar in genome size (~30 Mb) and possess more than 20 % species-specific genes. However, *N. oceanica* had smaller genome size but with higher protein count (~12 000) compared to *N. gaditana* (Pan et al., 2011; Radakovits et al., 2012; Vieler et al., 2012). Pan et al. (2011) pointed out that *N. oceanica* was haploid and asexual; only two orthologs of meiosis-specific proteins were identified in its genome. This was also confirmed by experiments with homologous recombination (Kilian et al., 2011).

From the palette of various algal transformation methods, such as agitation in the presence of glass beads or silicon carbide whiskers, biolistic microparticle bombardment, polyethylene glycol, *Agrobacterium tumefaciens*-mediated gene transfer and electroporation, only two last methods have been used in eustigmatophytes (Cha et al., 2011; Chen et al., 2008; Kilian et al., 2011). Efficient isolation of genetic transformants is largely facilitated by the use of selection markers, including antibiotic resistance (e.g. to zeocin) which are used much more rarely in microalgae than in plants, yeast or bacteria, because of the natural resistance of algae. Hence, there are other useful markers, such as fluorescent or biochemical markers, e.g. luciferase, β-glucuronidase, β-galactosidase, and green fluorescent protein (Cha et al., 2011; Radakovits et al., 2010).

On account of limited knowledge of gene functions among Stramenopiles species in general, Shi et al. (2008) constructed a cDNA library and carried out expressed sequence tag analysis of almost 2 000 nonredundant sequences in *N. oculata*. Then the construction and characterization of a normalized cDNA library followed (Yu et al., 2010). Among others unigenes encoding enzymes

involved in biosynthesis and storage of both FAs and PUFA were recognized. The first attempts of transformation of *N. oculata* have been reported after the enzymatic digestion of the cell wall (Chen et al., 2008). However, the lack of a selectable marker gene in the transformation construct necessitated PCR screening of hundreds of colonies to identify a few stable transformants. Plasmid phrYPGHc, containing the fish growth hormone cDNA driven by a heat shock protein 70A promoter and a RuBisCO SSU 2 promoter, was transferred into the protoplast of *N. oculata* by electroporation. Eventually, the result of the study was quite convincing – fish larvae grew twice or three times faster when fed with artemia incubated with transgenic microalgae (Chen et al., 2008).

After optimization, the voltage of electroporation used for intact *N. oculata* cells (not treated by emzymatic digestion) was higher (2 kV) than the one used to electroporate the protoplasts (1 kV). A higher voltage was observed to be harmful to the transformed cells (Chen et al., 2008). Disapprovingly, Kilian et al. (2011) and Radakovist et al. (2012) reported the highest transformation efficiency when using high electric field strength (11–12 kV/cm).

Transgenic transformation of *Nannochloropsis* sp. using *Agrobacterium tumefaciens* has been described in order to assess the efficacy of alternative natural phenolic compounds on transformation instead of acetosyringone (Cha et al., 2011). Cinnamic acid, vanillin and coumarin were found to induce higher percentages of transformations compared to acetosyringone. In addition, their price is lower compared to acetosyringone, which has been favoured in plant transformations for last few decades.

Kilian et al. (2011) were the first to perform high-efficiency homologous recombination in *Nannochloropsis* sp. by insertion of linear DNA transformation constructs into the nuclear genome using a selectable marker gene (e.g. *Sh ble* conferring resistance to zeocin). While targeting to genes encoding nitrate and/or nitrite reductases they generated knockouts for easy detection by growth on different nitrogen sources.

A hundred genes in *N. oceanica* are related to lipid metabolism (Vieler et al., 2012). Experiments with overexpression of some enzymes of FA biosynthesis (e.g.ACCase) or TAG assembly (e.g. G3PDH, PAP, DAGAT), or with the knockdowns (e.g. RNA silencing) of enzymes involved in lipid degradation or in other metabolic pathways influencing the accumulation of energy-rich storage compounds (e.g. starch) were performed mainly in *E. coli*, yeasts or plants bringing more or less successful results. Future targets for overexpression to increase TAG production in *Nannochloropsis* are phosphatidic acid phosphatase (PAP) and phospholipid:DAG acyltransferase (PDAT), which have only one homologue, alongside with carbonic anhydrases to improve carbon assimilation. In contrary TAG lipases and acyl-CoA oxidases as well as genes involved in gluconeogenesis are interesting targets for gene knockout or knockdown for the purpose of increased lipid production (Radakovits et al., 2012, 2010).

6 Conclusions

The topic of the potential use of microalgae in the production of biofuels has been highly discussed recently. Most of the works on that subject emphasize its importance and consider algae as an adequate substitution for the fossil fuels, possibly leading to the sustainability. Whether such a vision is really a feasible one we will see in the future. It still seems that there are much more questions, unresolved issues and doubts than convincing results. Nevertheless, the fact is that there has been a boom of research on the field of applied phycology nowadays not only due to biofuels industry, but also because of the investigation of algae usage in other applications, e.g. nutraceuticals, pharmaceuticals, cosmetics, aquaculture, bioremediations etc.

To introduce the biofuels production issue in detail has not been possible in here because of the limited space of this thesis. In fact, there are lots of methods and techniques involved, such as algae cultivation (with various designs and constructions of culture systems), harvesting and biomass concentration, processing of lipid extraction and finally biodiesel production. Thus, one can imagine that the most effective combination of different approaches has not turned up yet. In fact, this is the most difficult task to be done in this field, not just a subject of another thesis. The most problematic steps for energy sustainability are the most expensive processes, such as the dewatering and lipid extraction. It is unquestionable that, despite numerous start-ups claiming enormous productivities of biomass convertible into oil at low cost, microalgae oil is not a mature technology. It is still having a lot of drawbacks preventing the emergence of an economically viable algal biofuel industry. The main question still remaining is how to bridge the gap between the high current cost of algal biomass production (Rodolfi et al., 2009).

This bachelor thesis depicts the role of eustigmatophytes in the production of biofuels with consideration of the issue of various cultivation condition effects on lipid yields, plus it also briefly mentions the recent rapid progress in genetic engineering in eustigmatophytes. The following points have been discussed.

- The class Eustigmatophyceae has been briefly introduced by its morphological, ecological and molecular taxonomy-related features (Fig. 3).
- The lipid classes present in eustigmatophytes were described (Fig. 4), alongside with the fatty acid profiles with the focus on polyunsaturated fatty acids (Table 1, 2). The first review of fatty acid distribution in eustigmatophytes ever reported is provided herein.
- The nutrient and environmental factors that influence the lipid content and composition are also discussed so that to provide an instrument for the improvement of the production of desired compounds, or for optimization of productivity by different cultivation approaches.
- The process of (polyunsaturated) fatty acid biosynthesis is briefly outlined, with some steps in the pathways emphasized as for they could serve as a potential target for metabolic engineering.
- Eventually, the progress in genetic engineering techniques is summarized.

This work can contribute to the strain selection issue being a summary of FA distributions of the most of the reported eustigmatophytes. The aim was not to pick up the best alga for biofuels

production, but rahter to summarize the contemporary knowledge of the eustigmatophyte lipid contents since nothing like this has been done before. One can be confused by the high variability of the FA profile on both levels – intraspecific and interspecific (Table 1, 2). But eventually, there are some patterns which can be followed. The most promising strains showed up in *Monodopsis subterranea* for its high productivity in dense cultures, *Trachydiscus minutus*, which prefers lower light intensities, is therefore more suitable for cultivating in temperate regions, and of course *Nannochloropsis,* the most investigated eustigmatophyte ever, which has the potential to emerge as a model organism for biofuels technology research. Moreover, the strains with higher proportin of saturated and monounsaturated fatty acids to polyunsaturated fatty acids could be selected in order to produce the biofuels compatible with the engines used at present according to existing standards.

To compare fatty acids distributions in different algae has been quite a difficult task, mainly because of the fact that each author collective has used different methods of either cultivation or lipid extraction – both of which strongly affect the result of lipid composition detected. Therefore, a complex and standardized lipid examination of the whole class Eustigmatophyceae (Fig. 3) will be needed in the future so that the trustworthy comparison of their lipid contents would be possible to make. Furthermore, a pattern in fatty acid composition, which would reflect phylogenetic relations in the Eustigmatophyceae, could be examined.

This work summarizes most of the studies on environmental and nutritional effects on eustigmatophytes which often provide contradictory inferences, therefore the attempt to discuss their inconsistencies has been performed here, or just the overview of the development of this particular field has been recounted. Further transcriptomic, proteomic and metabolomic investigations are needed in order to determine the exact mechanisms of lipid accumulation during nutrient deprivation. Moreover, there are also many unresolved issues in lipid synthesis itself, e.g. trafficking pathways – how the lipids synthesized in cytosol get to the chloroplast.

Consequently, there is also an approach of genetic engineering trying to control algal lipid and fatty acid production. Because none of the algae currently in use has a history of domestication, and bioengineering of algae is still in its infancy, therefore there is a need to develop algal strains adapted to cultivation for industrial large-scale production of desired compounds. Moreover, significant advances in the development of genetic manipulation tools have recently been achieved with microalgal model systems and are being used to manipulate central carbon metabolism in these organisms. Nevertheless, further research is needed in this area, as well (Radakovits et al., 2012, 2010).

7 References

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